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Article Addendum

Growing in darkness

The etiolated lupin hypocotyls

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Epigeal germination of a dicot, like lupin (*Lupinus albus* L.), produces a seedling with a characteristic hypocotyl, which grows in darkness showing a steep growth gradient with an elongation zone just below the apex. The role of phytohormones, such as auxin and ethylene, in etiolated hypocotyl growth has been the object of our research for some time. The recent cloning and expression of three genes of influx and efflux carriers for polar auxin transport (*LaAUX1*, *LaPIN1* and *LaPIN3*) reinforces a previous model proposed to explain the accumulation of auxin in the upper growth zone of the hypocotyl.

Most plants show a typical axial, polar and branched (dendritic) morphology to compensate for their immobility by optimally exploiting the resources available in a limited environment.

From Julius von Sachs[1] to Tsvi Sachs[2] many plant physiologists sought to explain how the axis is maintained and what type of signals are interchanged between poles. It was demonstrated that auxins were the determining factors in maintaining the polarity in shoots and roots and a reductionistic approach leads to conclude that such polarity had to be established at the cellular level. A chemiosmotic theory was then proposed, which implied an asymmetric distribution of efflux carriers at the bottom of a cell, linked to pH gradients to maintain different undissociated/dissociated forms of auxin separated between apoplast and symplast spaces.[3]

In recent years, the use of *Arabidopsis thaliana* as a plant model has given additional support to the hypothesis that polar auxin transport is restricted to certain cells and mediated by influx (AUX1 and LAX1-4 proteins) and efflux carriers (PIN1-8 proteins).[4-6] Currently, we have a good idea of the topology of Arabidopsis carrier distribution, especially in roots.[4,5] Additional (MDR/PGP)[7] or parallel (TRH1)[8] components of the transport system are now emerging.

However, while accepting the enormous advances and contributions to plant science provided by the use of *Arabidopsis thaliana*, we remain true (loyal) to the particular model adopted by the Department of Plant Biology, University of Murcia (Spain) in the 1970's: the hypocotyl of lupin seedlings cultivated in darkness. In such conditions, the organ grows heterotrophically and longer than in light.

The cotyledons and meristem at the top supply nutrients and hormones in a basipetal direction.

The hypocotyl is a cylindrical column, with a radial symmetry that clearly shows differentiated tissues: epidermis, cortex, vascular cylinder and pith. Its size allows surgical separation of the tissues using suitable glass capillaries.

At the beginning lupin was chosen because it had higher IAA-oxidase activity than pea, bean, oat or barley seedlings. At the time, it was thought that growth was mainly controlled through auxin catabolism (a fruitful line involving peroxidases was developed later). However, the etiolated hypocotyl was soon adopted preferentially by our group because of its qualities as a model for studying the relationship between hormone levels (auxin and ethylene) and growth. Our Portuguese colleagues have also used lupin as a model with successful results.[9]

Bellow, we detail the landmarks of our research to date. Hypocotyl growth shows a characteristic pattern. Unlike plants grown in the light, in which all the cells along the hypocotyl elongate continuously throughout the growth period,[10,11] there is a steep growth gradient in the dark with an elongation zone just below the apex[12] (see Fig. 1 for details). This cell growth pattern in etiolated hypocotyls was described in lupin and then in Arabidopsis.[11] In this pattern, it is important to note that there is compensation along the organ between the cell diameter and the cell wall thickness. Once the cell growth pattern was known, we investigated its relation with the level of two phytohormones, auxin and ethylene, which might participate in the growth regulation. Special attention was paid to the distribution of endogenous IAA and its relation with growth. The results showed good correlation between the auxin levels and the cell size.[13,14] Auxin from the apex appears to be responsible for hypocotyl growth, since decapitation of seedlings strongly reduced growth, which was restored after the application of exogenous IAA to the cut surface.[15] In light of the fact that growth depended on auxin from the apex, we investigated the nature of the auxin transport and demonstrated that this transport is polarized and sensitive to inhibition by specific

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inhibitors of *polar auxin transport* (PAT) such as 2,3,5-triiodobenzoic acid and 1-*N*-naphthylphtalamic acid (NPA).[16,17] Basipetal PAT mainly occurred in the stele,[15] while cells in the epidermis and outer cortex are the limiting factor in auxin-induced shoot growth.[18-20] The finding that during PAT auxin can move laterally from transporting cells in the stele to the outer tissues of the elongation zone[15] could explain the apparent conflict between the localization of PAT and the auxin target cells for elongation. In fact, epidermal cells acted as a sink for *lateral auxin movement* (LAM).[17]

If PAT provides the auxin for growth and elongating growth is restricted to the apical region in etiolated hypocotyls, the question is: how does auxin accumulate in the elongation region?

In a former study, we proposed that variations in auxin transport along actively growing lupin hypocotyl could produce such accumulation.[21] Recently we extensively studied the variation of PAT along the lupin hypocotyls in seedlings of different ages, finding that certain parameters of PAT, such as transport intensity, polarity (basipetal vs acropetal) and sensitivity to NPA inhibition, showed a good correlation with the distribution of growth along the hypocotyl and its variation with ageing.[22] These results suggest that a basipetally decreasing gradient in PAT along the hypocotyl may be responsible for the auxin distribution pattern controlling growth, since the existence of such a PAT gradient might generate the so-called *barrier effect*, which could produce an auxin gradient along the hypocotyl, the auxin content being higher in the apical elongation zone. To investigate whether these PAT variations can be explained in terms of auxin carrier distribution, we isolated three genes coding for auxin influx (*LaAUX1*) and efflux (*LaPIN1* and *LaPIN3*) carriers, and studied their expression in different tissues along the hypocotyl at different ages.[23] The expression of *LaAUX1* and *LaPIN3* occurred both in the stele and in the outer tissues, while the expression of *LaPIN1* was restricted to the stele and showed a basipetally decreasing gradient along the hypocotyl. The decisive role ascribed to PIN1 in polar auxin transport due to its localization in the basal end of transporting cells,[24] and the existence of such a gradient in the expression of *LaPIN1* support the hypothesis of a *barrier effect* (generated by decreasing auxin transport) previously proposed as being responsible for the auxin gradient which controls the growth pattern in etiolated lupin hypocotyls.

The acid-growth theory of auxin action was also tested, observing that the elongation growth of etiolated hypocotyl segments of lupin was stimulated by acid pH and IAA. Both factors stimulated growth in a more than additive way, suggesting a synergistic action between them.[25] The recent finding of a soluble auxin receptor (intracellular) reinforces the interest of the above study (which has remained a "sleeping beauty") because pH affects IAA uptake.

There are still several questions that must be answered before we can fully understand the growth pattern exhibited by etiolated lupin hypocotyls. Thus, as regards the cause of the PAT gradient, other factors besides the LaPIN1 gradient must be considered. For example, auxin carriers such as some phosphoglycoproteins (PGP), are also expressed differentially along the Arabidopsis hypocotyl and specific PIN-PGP pairings influence PAT by modulating the rates of cellular auxin movement.[7] The pathway (symplast or apoplast) and mechanism of LAM remains unknown. Although alternative mechanisms have been proposed,[26] a previous study in lupin[15] suggested that LAM is a diffusive process and that the IAA metabolism

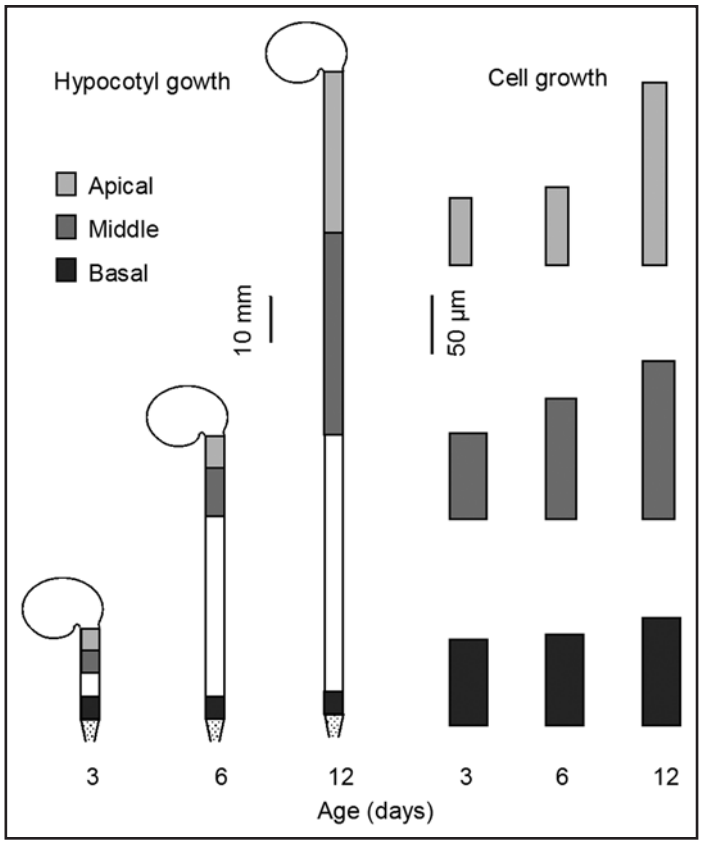

Figure 1. Distribution of growth and cell size along the hypocotyl in etiolated lupin seedlings. At 3 d, hypocotyls were marked with ink, delimiting four 5-mm long zones including the apical, middle and basal zones. The hypocotyl growth ceased at day 12 and almost no growth was observed in the basal zone after day 3. From 3 to 6 d the growth was localized between the apical and basal zones, while most growth occurring from 6 to 12 d was localized in apical and middle zones. The cell size represents the cell length and cell diameter (the cell wall excluded) and corresponds to the second cell layer of cortex near the vascular cylinder. Similar results were obtained in cells from epidermis and pith. In each zone the cell length increased and the cell diameter showed little change during hypocotyl ageing. The final size at the end of the growth period varied along the hypocotyl, the cells becoming shorter and broader from the apical to the basal zones. In spite of the fact that cell diameter increased basipetally, no significant variation in hypocotyl diameter was found along the organ during the growth period. A morphometric study revealed that cell wall thickness in the apical cells was twice that in the basal cells at the end of the growth period i.e., the thinner apical cells had thicker cell walls, which may help explain the consistency of hypocotyl diameter along the organ.

observed in the outer tissues might generate the radial gradient of auxin necessary for the maintenance of its lateral flow. It is thought that this metabolism of IAA occurs once the hormonal action is completed.[25,27] Although NPA does not inhibit LAM, the involvement of auxin efflux carriers cannot be discarded. In fact, the role of PIN carriers in lateral auxin transport towards and from the stele has been described in the root.[28] Other phytohormones besides auxin can modulate hypocotyl growth. Thus, the ethylene production rate, the 1-aminocyclopropane-1-carboxylic acid (ACC) content and the ACC oxidase activity decreased along the hypocotyl during the hypocotyl growth period.[29] Sensitivity to exogenous ethylene varied during growth, the young apical region being less sensitive than the older basal region.[30] Ethylene modified the cell growth pattern

in the different tissues.[31] The ethylene-induced lupin hypocotyl thickening was irreversible and mainly due to an increase in cell diameter. However, the inhibition of hypocotyl elongation produced by ethylene was reversible and involved irreversible inhibition of cell division and, paradoxically, stimulation of cell elongation to produce cells longer than those of the control.[32]

Studies in Arabidopsis showed that the hypocotyl growth in both light- and dark-grown plants is a process driven by cross-talk between multiple hormones. Interactions between auxins, ethylene, gibberellins and brassinosteroids have been described.[33,34] We think that the etiolated lupin hypocotyl remains a suitable model for confirming some of these results and for opening up new approaches in phytohormone research.

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